IN VITRO IMMUNOMODULATORY EFFECT OF *Andrographis paniculata* EXTRACT ON CYTOKINE GENE EXPRESSION AND CELL PROLIFERATION IN HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

A PROJECT REPORT

Submitted by

AISHWARYA. S

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SRI VENKATESWARA COLLEGE OF ENGINEERING

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BONAFIDE CERTIFICATE

Certified that this project report **"INVITRO IMMUNOMODULATORY EFFECT OF *Andrographis paniculata* EXTRACT ON CYTOKINE GENE EXPRESSION AND CELL PROLIFERATION IN HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS"** is the bonafide work of "**AISHWARYA. S (Reg. No. 2127180201003)**" who carried out the project work under my/our supervision.

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EXTERNAL EXAMINER

ABSTRACT

Andrographis paniculata is a medicinal plant that contains a natural compound that has various biological activities comprising anti-tumor, anti-microbial, and immunomodulatory properties. Until now, immune responses promoted by andrographolide is still not clear. The present study aims to isolate and assess the immunomodulatory effects of andrographolide from the *A. paniculata* extract in human PBMC. An efficient ultrasound-assisted extraction of andrographolide from the *A. paniculata* in water was performed at different sonication durations (2, 4, 6, 8, and 10 min). Furthermore, the highest yield of andrographolide was 99.34 μg/mL for 4 min and the lowest yield of andrographolide was 25.073 μg/mL for 8 min. The identification of extracted andrographolide was confirmed by HPLC by comparing it with standard andrographolide. The structures of the extracted andrographolide were elucidated by FTIR, NMR, and UV spectroscopy. PBMC were isolated through ficoll technique, were incubated with different concentrations of the extracted andrographolide (1-10, 15, 20, 25, 30, 35, 40, 45, and 50 μg/mL) for 24 and 48 hr, and was performed cytotoxicity and lymphoproliferation assay against Con-A and LPS. The extracted andrographolide did not exhibit any significant cytotoxicity against PBMC. The andrographolide extract had more than 85 % cell viability at 50 μg/mL after 24 and 48 hr of exposure. Andrographolide from the *A. paniculata* extract was found to stimulate proliferation of human PBMC. A maximum proliferation (≈50%) was observed at 10 μg/ml concentration after 24 and 48 hr of exposure. Therefore, andrographolide from the *A. paniculata* extract promotes immunomodulation in human PBMC. Further investigation should be considered on the effect of extracts on other immune parameters such as macrophage activity, NK cell activity including cell signaling and cytokine production.

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AISHWARYA. S

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LIST OF ABBREVIATIONS

A. paniculata	*Andrographis paniculata*
Abs	Antibodies
Con A	Concanavalin-A
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immuno Sorbent assay
FTIR	Fourier transform infrared
HPLC	High-performance liquid chromatography
IFN-gamma	Gamma interferon
IgA	Immunoglobulins-A
IgD	Immunoglobulins-D
IgE	Immunoglobulins-E
IgG	Immunoglobulins-G
IgM	Immunoglobulins-M
Igs	Immunoglobulins
IL-12	Interleukin-12
IL-4	Interleukin-4
min	Minutes

NADP	Nicotinamide adenine dinucleotide phosphate
NK	Natural killer
NMR	Nuclear magnetic resonance
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
Ppm	Parts per million
RPMI	Roswell park memorial institute
SE	Standard mean error
UV	Ultraviolet
VA	Viscum albumin
Xg	Relative centrifugal force

LIST OF SYMBOLS

α	alpha
β	beta
℃	degree Celsius
γ	gamma
g	gram
g/mL	gram/millilitre
g/mol	gram/mole
hr	hours
µg/mL	microgram/millilitre
µl	microlitre
mg	milligram
ml	millilitre
mVs	millivolts
cm^{-1}	reciprocal centimeter
RPM	revolutions per minute
v/v	volume/volume
w/v	weight/volume

CHAPTER 1

INTRODUCTION

1.1 IMMUNE CELLS

An immune cell is a kind of immune system that aids the body in fighting off pathogens and other disorders. The white blood cells are often referred to as immune cells. The white blood cells are on constant patrol. They regularly circulate in the blood. when they find a target, they signal to the other cells and multiply.

Immune cells develop from bone marrow stem cells into various types of white blood cells. Macrophages, mast cells, eosinophils, dendritic cells, basophils, monocytes, neutrophils, lymphocytes, and natural killer cells are some of the several types of white blood cells (B cells and T cells). The immune cells are the one that protects our body against bacteria and viruses and keeps our body safe by producing antibodies against pathogens.

1.2 IMMUNE SYSTEM

For human survival, our immune system is vital. Our body would be vulnerable to bacteria, viruses, parasites, and other pathogens if we didn't have an immune system. As we float over a sea of germs, it is our immune system that keeps us healthy.

The immune system can be stimulated by a variety of substances that the body does not recognize as its own. These are known as antigens. Antigens

include proteins found on the surfaces of bacteria, fungi, and viruses. When these antigens bind to particular receptors on immune cells (immune system cells), a cascade of events occurs in the body. When the body first comes into contact with a disease-causing bacterium, it normally saves information about the infection and how to combat it. If it comes into contact with the germ again, it recognizes it immediately and can begin fighting it more quickly. (Anastassova-Kristeva,2003).

1.2.1 Types of immune system

There are two types of the immune system

1. Innate immune system
2. Adaptive immune system

1.2.1.1 Innate immune system

This is the first line of defence. Invaders such as viruses, bacteria, parasites, and poisons, as well as wounds and trauma, are detected early by the innate immune system. The innate immune system stimulates cells to attack and eliminate the outsider, or to initiate repair when these agents or events are detected, while also informing and influencing the adaptive immune response that follows this first line of defence (Medzhitov *et al.*, 2000).

Unlike adaptive immune responses, which are specific to a certain pathogen, innate immune responses are not. They rely on a collection of proteins and phagocytic cells that recognize infections by their conserved traits and become activated immediately to assist kill intruders. Unlike the adaptive

immune system, which evolved less than 500 million years ago and is only present in vertebrates, innate immune responses have been discovered in both vertebrates and invertebrates, as well as in plants, and the underlying mechanisms that regulate them have remained unchanged. Invertebrate's innate immune responses are also required to activate adaptive immunological responses (Turvey *et al*., 2010).

1.2.1.2 Adaptive immune system

The innate immune system activates adaptive immune cells, which are the second and most particular line of protection.

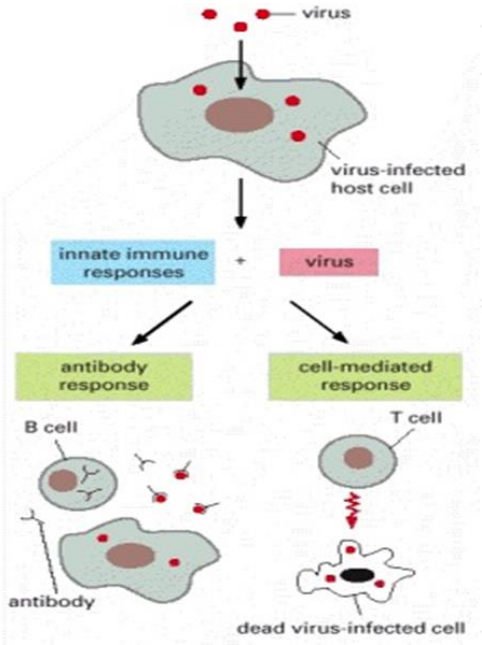


Figure 1.1: Immune system response to the virus

Lymphocytes are responsible for both types of reactions. The lymphocytes in this image are responding to a viral infection. B cells release antibodies that neutralize the virus in one type of response. T lymphocytes kill virus-infected cells in the other, cell-mediated response (Chaffey *et al.*, 2003).

After recognizing the invader, the cells can grow and fight it, resulting in disease recovery and protection from recurrence (Bonilla *et al*., 2010). There are two main classes of the adaptive immune response (Figure 1.1).

- The cell-mediated immune response is carried out by T cells.
- The humoral immune response is controlled by activated B cells and antibodies.

1.3 COMPONENTS OF THE IMMUNE SYSTEM

The immune system is made up of several components that work together to protect the body from foreign intruders. The bone marrow and thymus are two of the most important components of the immune system. Because all of the body's blood cells (including T and B lymphocytes) originate in the bone marrow, it is critical to the immune system. T cells migrate to the thymus, while B lymphocytes stay in the marrow to mature.

After maturing in the thymus and bone marrow, T and B lymphocytes migrate to the lymph nodes and spleen, where they stay until the immune system is activated. Lymph nodes can be found all over the body. The spleen is placed behind the stomach and under the diaphragm in the upper left portion of the abdomen.

The spleen's primary role is to filter blood. Damaged red blood cells are broken down by macrophages (large white blood cells specialized in absorbing and digesting cellular debris, infections, and other foreign substances in the body) in the spleen, while healthy red blood cells pass through the spleen

effortlessly. The spleen is where platelets and white blood cells are stored. The spleen helps and guides the immune system to recognize the foreign particle that entered the body (Janeway *et al*., 2001).

1.4 CELLS OF THE IMMUNE SYSTEM

All immune system cells develop from hematopoietic stem cells in bone marrow, which eventually differentiate into myeloid and lymphoid progenitors (Figure 1.2). Each of them divides into a distinct cell type. Natural killer cells are developed from both myeloid and lymphoid progenitor.

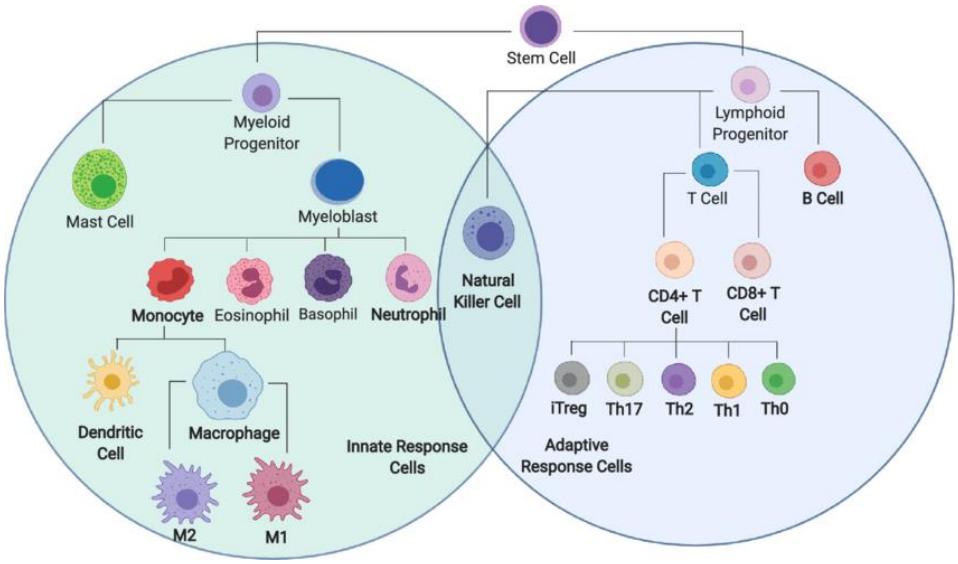


Figure 1.2: Cells of the immune system developed from hematopoietic stem cells.

Immune system cells are shown as a lineage tree. Hematopoietic stem cells give rise to immune cells (HSCs). HSCs develop into lymphoid and myeloid progenitors, which branch out into more specialised cell types involved in adaptive and innate immunity. The primary immune cell subsets are depicted in this diagram, with arrows indicating lineage links. (Torang *et al*., 2019)

Myeloid cells identify pathogen-associated molecular patterns, which triggers a signaling cascade that culminates in the release of cytokines like interferons to help fight infections. Lymphoid cells migrate to the thymus, where they go through a series of screening stages to ensure that only functional, non-autoreactive T cells make it into circulation (Parker *et al*., 2017).

1.4.1 Cells of innate immunity

Myeloid Progenitor cells make up the majority of the innate immune system.

- *Neutrophils*-Neutrophils are granulocytic leukocytes with multilobed nuclei. They are the first cells to activate at the site of tissue injury. The number of neutrophils in the circulation increases during sickness, and they are principally responsible for the increased number of white blood cells associated with various illnesses.
- *Eosinophils*- They are granulocytic leukocytes that have a kidney-shaped, lobed nucleus. They destroy infections by releasing the contents of their granules. They create several growth factors and cytokines and aid other immune cells.
- *Basophils*- They are the biggest granulocytic leukocytes having a bilobed nucleus. They have histamine-rich granules and participate in inflammatory reactions.
- *Monocytes*-They are phagocytic cells found in the circulation that change into macrophages when they reach tissues. Macrophages and Dendritic cells are produced by monocytes, which are the biggest white blood cells.

- *Dendritic cells*- These cells can be found in a variety of tissues, including the skin, lungs, and intestines. They release cytokines and deliver antigens to B and T cells (Parker *et al*., 2017).
- *NK cells (Natural Killer)*-These cells develop from both Myeloid and Lymphoid progenitors. NK cells are generated in the bone marrow and are detected in relatively small levels in the circulation and tissues. They are cytotoxic cells with tiny granules containing perforins and granzymes (Cichocki *et al*., 2019).

1.4.2 Cells of adaptive immunity

Lymphoid progenitor cells make up the majority of the adaptive immune system. B-Cell Progenitor- The lymphocytes arise and divide into plasma cells throughout the bone marrow, eventually developing into immunoglobulins (antibodies).

The B cell differentiates into plasma cells and memory cells.

- *Plasma cells*-These cells develop from B-cells and are the components of serum and immunoglobulin discharges.
- *Immunoglobulins*-The adaptive humoral immune response is controlled by immunoglobulins (Igs) or antibodies (Abs) (Mix *et al*., 2006)
  - There are five classes of immunoglobulin
    - *IgM*-generated mostly in the first immunological response to pathogenic pathogens or antigens
    - *IgG*-highly protective and activates the classical pathway.
    - *IgA*-epithelial surfaces of the respiratory, digestive, and the genitourinary system is protected by the mucosa membrane

- *IgE*-mediates immediate hypersensitivity by releasing mediators from mast cells.
- *IgD*-There is a little quantity of this protein in the serum, and its activity against infections is unclear. It is required for antigen-stimulated lymphocyte differentiation (Vaillant *et al*., 2021).

1.5 EFFECT OF HERBAL MEDICINE ON THE IMMUNE SYSTEM

Herbal medications, in combination with the human body's immune system, tend to promote an equitable detoxification process. Herbal remedies are made from plant stuff that is largely safe and easily digestible by humans (Maqbool, 2019). The benefits of utilizing herbal remedies include the fact that they are quite easy to swallow and may be used regularly. Various herbal remedies have been discovered to alter innate and acquired immune system components. Indeed, plants produced from secondary metabolites in natural goods can be the lead molecules for the future development of immunomodulators for therapeutic application, based on a thorough understanding of the varied immunomodulatory actions of herbal plants.

Based on investigations done on numerous animal models, several immunomodulators have been recommended for various allergic disorders such as asthma, allergy, and other diseases (Sharma *et al*., 2017).

1.5.1 Herbal market

The herbal medications industry has grown significantly over the last several decades as people have become more conscious of the escalating expense of synthetic pharmaceuticals as well as the adverse effects associated with their usage. Plants have long been used as a traditional remedy in India,

and they form an essential part of the country's healthcare system. India is abundant in therapeutic and ayurvedic plant resources, which serve both as a raw material supply for chemotherapeutics used in the indigenous system of medicine, as well as a source of health care for rural India.

Most practitioners in Indian medical systems design and deliver their formulas, necessitating meticulous documentation and study. Due to the rising prevalence of adverse medication responses and the cost burden of the contemporary system of medicine, public, academic, and compelling state interest in traditional treatments is expanding dramatically (Seth *et al*., 2004).

There are an estimated 5,00,000 plant species on the planet today, with the number fluctuating depending on whether subspecies are included. It is worth noting that plant treatments are still used by 80 percent of the world's population. Plant medicinal knowledge has been passed down in numerous regions and civilizations. Medicinal plants contain variable amounts of chemicals or antibiotics, which have a direct effect on physiological activity (Sharma *et al*., 2017).

1.6 *Andrographis paniculata*

In modern pharmacology, *A. paniculata* has been used to stimulate the immune system and treat myocardial ischemia, pharyngotonsillitis, and respiratory tract infection (Dai *et al.*, 2019). *A. paniculata* (Burm. f.) Nees (Acanthaceae) a medicinal plant native to India, China, and Taiwan is a bitter-tasting medicinal herb. The leaf and stem of *A. paniculata* may operate by

stimulating the immune system. It may also hinder flu viruses from attaching to bodily cells (Akbar *et al*., 2011).

A. paniculata (Figure 1.3) is a branched, upright, herbaceous annual that grows in hedgerows on plane lands, hill slopes, waste ground, farms, moist environments, seashores, and roadsides. It can even be grown in a garden. For their good development, moist gloomy areas, woodlands, and wastelands are preferable (Okhuarobo *et al*., 2014). The taxonomy of the plant is represented in Table 1.1.

Figure 1.3: *Andrographis paniculata*

A medicinal herb, grown in Madhavaram, Chennai taxonomically verified at the herbarium, University of Madras, Guindy.

1.6.1 Morphological Characteristics

- It is a 30-90 cm tall upright annual herb with a quadrangular upper stem and a somewhat spherical lower stem.
- Leaves are sessile or subsessile, linear-lanceolate or lanceolate, 3-8 cm long, acute, glabrous, or minutely puberulous beneath and base cuneate, with a slightly undulating edge (Jayakumar *et al*., 2013).
- The slender stem is dark green in colour and has a square cross-section with longitudinal furrows and wings along the angles.

1.6.2 Taxonomy of Plant

Table 1.1: Tabulation of Taxonomy of plant *A. paniculata*

Kingdom	Plantae, Plants
Subkingdom	Tracheobionta, Vascular plants
Super Division	Spermatophyta, Seed plants
Division	Angiosperma
Class	Dicotyledonae
Sub Class	Gamopetalae
Series	Bicarpellatae
Order	Personales
Tribe	Justicieae
Family	Acanthaceae
Genus	*Andrographis*
Species	*Paniculata*

1.6.3 Ethnomedicine

A. paniculata extract is used to increase immunity, treat liver diseases, intestinal complaints in children, colic discomfort, common cold, and upper respiratory tract infection. It is often used to treat the common cold, osteoarthritis, throat and tonsil infections, and ulcerative colitis, a form of bowel disease. It is also used to treat a variety of other diseases, but there is no good scientific evidence to back up these claims (Jarukamjorn *et al*., 2008).

It is widely utilized in ayurvedic treatment, an ancient Indian medicine system. As mentioned in ancient ayurvedic literature, the herb can be used to treat neoplasm. In Chinese medicine, the aerial section of *A. paniculata* is often employed. *A. paniculata* cools and soothes internal heat, inflammation, and discomfort, and is utilized for detoxication, according to Chinese medicine philosophy (Chao *et al*., 2010). It has been found to exhibit antityphoid, antifungal, antihepatotoxic, antibiotic, antimalarial, anti-thrombogenic, anti-inflammatory, and antitumor activities (Chandrasekaran *et al.*, 2011).

1.7 ANDROGRAPHOLIDE

In terms of bioactive characteristics and abundance, andrographolide is the most abundant single component recovered from *A. paniculata*. In plant leaves, there is a substantial amount of andrographolide (0.054 - 4.686%). Andrographolide is a labdane diterpenoid found mostly in *A. paniculata's* aerial portions. Andrographolide's chemical formula and weight are $C_{20}H_{30}O_5$ and 350.4 g/mol, respectively.

The bitter flavor of this major bioactive component of *A. paniculata* earned it the name "King of Bitters" (Mishra *et al*., 2007). Andrographolide can be extracted from *A. paniculata* using organic solvents such as ethanol, chloroform, ether, acetone, and dimethyl sulfoxide. Since andrographolide is a secondary metabolite, its activity is influenced by both environmental and seasonal factors. There is variation in andrographolide concentration in different parts of plants and different geographic regions. The plant comes from the family of Acanthaceae (which includes 40 species) and grows up to a meter tall, usually half a meter.

It has been shown to have numerous pharmacological properties such as anticancer, antidiarrhea, anti-HIV, antihyperglycemic, anti-inflammatory, anti-microbial, antioxidant, cardiovascular, cytotoxic, hepatoprotective, immunostimulatory, and sexual dysfunction therapies (Dai *et al*., 2019). In experimental models of asthma, stroke, and arthritis, as well as individuals with upper respiratory tract infections, andrographolide, and its derivatives have been demonstrated to have anti-inflammatory properties.

Andrographolide lessens the production of cytokines, chemokines, lipid mediators, nitric oxide, and adhesion molecules through inhibition of the nuclear factor (NF)-κB signaling pathway. Literature reports demonstrate that andrographolide and its derivatives have a wide range of biological activities but are extensively studied for their anticancer properties (Kumar *et al*., 2004). This compound exhibits multiple pharmacological properties as well as being a potential chemotherapeutic agent.

1.7.1 Plant parts used

The whole plant has been used to treat diarrhoea, malaria, and respiratory infections, as well as an antidote for snake bites and toxic stings from various insects. While the entire plant is beneficial, the leaves contain the most valuable terpenoids, including andrographolide, followed by the stem and roots, and the seeds have the fewest (Mishra *et al*., 2010). All sections of the plant, however, are recognized to have some form of bioactivity (Dua et al.,2004).

1.7.2. Bioactive constituents

A variety of compounds are found in AP extract, including diterpenoids, diterpene glycosides, lactones, flavonoids, and flavonoid glycosides. (Li *et al*., 2007).

Andrographolide, 14-deoxy11,12-didehydroandrographolide(14-DDA),14-deoxyandrographolide (14-DA), Andrograpanin, neo andrographolide, 14-deoxy-14,15-didehydroAndrographolide, iso andrographolide, and 14-acetyandrographolide are some of the bioactive chemical constituents isolated from the extract of *A. paniculata* (Chao *et al*., 2010). The properties of these compounds are shown in Figure 1.5.

Andrographolide (Figure 1.4) is a multifunctional natural moiety with three hydroxyl groups (primary, secondary, and allylic alcohol) as well as an exocyclic double bond and lactone ring. In terms of stereochemistry, Andrographolide has two cyclohexane rings in the chair conformation, a -lactone bridge by 12 in E-configuration, and the -lactone ring in twisted conformation. (Chao *et al*., 2010).

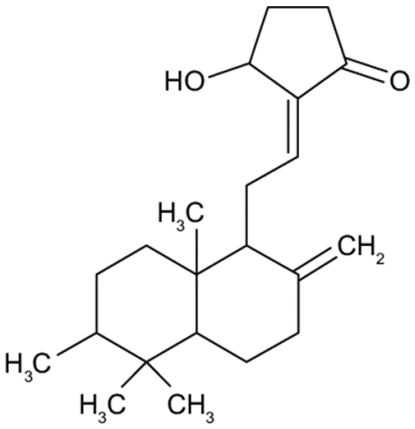
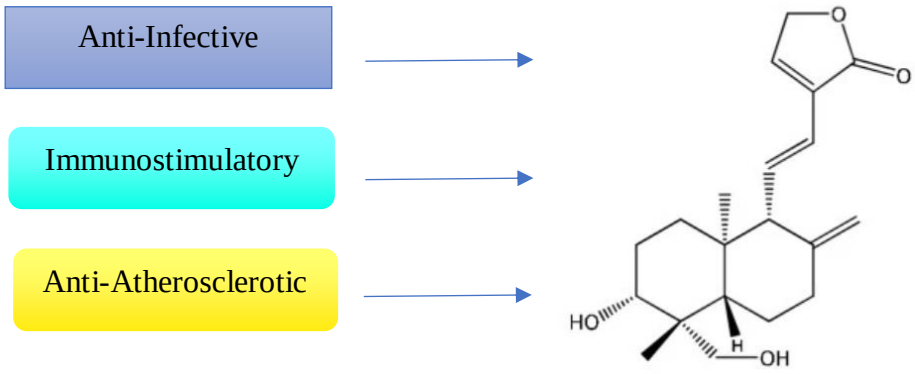


Figure 1.4: Chemical structure of andrographolide

1.7.3 Bioactive constituents and properties

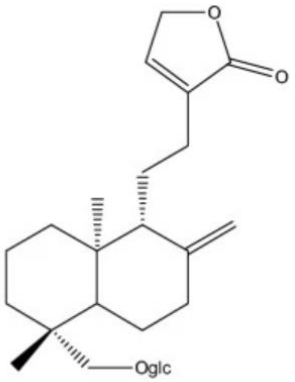


A

Anti-Infective

Immunostimulatory

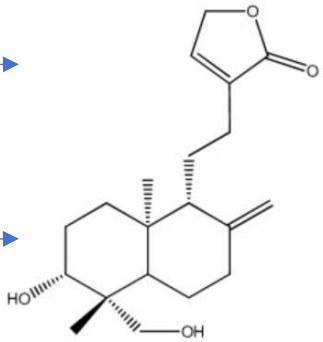
Anti-Hepatotoxic



B

Immunomodulatory

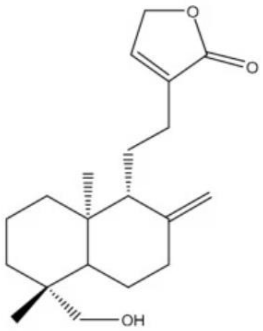
Anti-Atherosclerotic



C

Anti-inflammatory

Anti-infective



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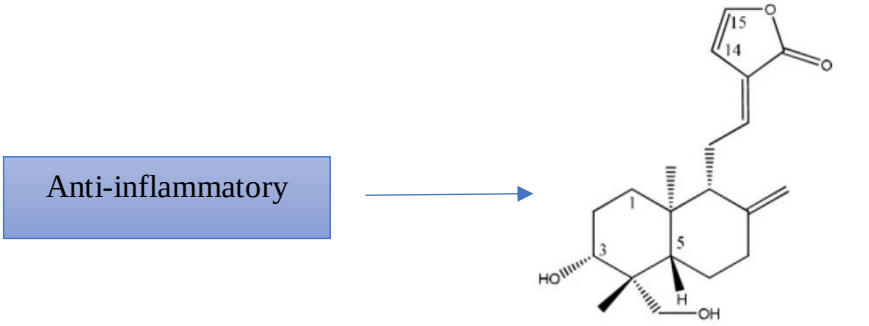
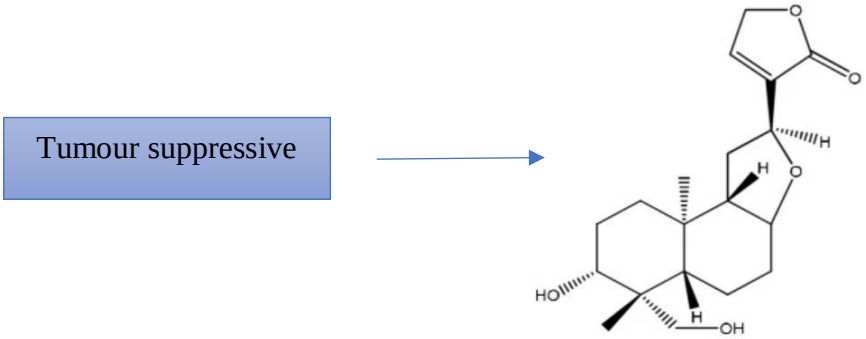


E



F

Figure 1.5: Bioactive compounds of *A. paniculata*

(A) 14-deoxy-11,12-didehydroandrographolide, (B) Neoandrographolide, (C) 14-deoxyandrographolide, (D) Andrograpanin, (E) 14-deoxy-14,15-dehydroandrographolide, (F) Isoandrographolide.

The structure and properties of the bioactive compounds of *A. paniculata*

CHAPTER 2

LITERATURE SURVEY

2.1 Pharmacological activities of *A. paniculata* and its major compound andrographolide

Dai *et al.*, 2019 thoroughly studied *A. paniculata* and andrographolide's therapeutic potential, with an emphasis on mechanisms of action and clinical application, and the structure-activity connection of andrographolide and derivatives was extensively explored.

2.2 Isolation and identification of bioactive compounds in *A. paniculata*

Chao *et al.*, 2019 examined *A. paniculata's* ingredients and pharmacological qualities, along with its chemical components, biological activities, and potential processes. This study focused on anti-inflammation, anti-cancer, immunomodulation, anti-infection, antihepatotoxicity, anti-atherosclerosis, anti-diabetes, and anti-oxidation properties of bioactive compounds in *A. paniculata*. It was concluded that in terms of bioactive characteristics and quantity, andrographolide was the most abundant component recovered from *A. paniculata*.

2.3 Harnessing the medicinal properties of *A. paniculata* for diseases

Okhuarobo *et al.*, 2014 phytochemical investigation found that the aerial sections of this species include diterpenoid lactones, which are the primary phytochemical elements, as well as flavonoids. The roots have yielded a variety of chemicals, including xanthones, uncommon noriridoids, and trace/macro elements. This plant's extracts, formulations, and pure components

have been demonstrated to exhibit biological actions such as anti-microbial, anti-inflammatory, antioxidant, anti-diabetic, cytotoxicity, immunological modulatory, liver enzyme modulatory, antimalaria, anti-angiogenic, and hepato-renal protective function. Pure chemicals produced from this plant, especially the bitter andrographolide, have the most promising biological actions. This review has offered a thorough understanding of *A. paniculata's* phytochemistry, therapeutic applications, and pharmacology.

2.4 Immunomodulatory activity of andrographolide on macrophage activation

Wang *et al.*, 2014 studied andrographolide was employed in vitro to modify LPS-induced classical activated (M1) or IL-4-induced alternative activated (M2) macrophages, as well as in vivo to influence the humor immunological response to HbsAg. In vitro, andrographolide suppressed either LPS or IL-4-induced macrophage activation, inhibited both M1 and M2 cytokine expression, and lowered the IL-12/IL-10 ratio. Andrographolide was shown to affect the production of antibodies by influencing macrophage phenotypic polarization and Ag-specific immune regulation.

2.5 Antimicrobial activity of *A. paniculata* extracts and andrographolide

Xu *et al.*, 2006 tested an aqueous and two ethanolic extracts of *A. paniculata*, as well as andrographolide, an active principle of *A. paniculata*, were for antimicrobial activity against nine bacterial species. Only two human pathogens, were shown to have direct antibacterial action against *A. paniculata* extracts at all doses tested. However, no antibacterial activity of andrographolide was identified against any of the pathogens tested in this study. Given that results of TLC revealed that andrographolide was present in all *A. paniculata* extracts, antibacterial activity was attributable to additional active

components present in the extracts employed in this study.

2.6 Antiatherogenic molecular mechanisms of andrographolide

Al Batran *et al.*, 2014 tested andrographolide for its antiatherogenic effect against *Porphyromonas gingivalis*-induced atherosclerosis in white New Zealand rabbits. Andrographolide has been shown in research to reverse biochemical abnormalities in the liver and kidneys. Andrographolide inhibited hyperlipidemia while decreasing oxidative stress indicators (MDA and nitrotyrosine) in rabbit serum and aortic utilized. Furthermore, andrographolide demonstrated antioxidant enzyme activity by boosting blood levels of SOD, CAT, GPx, and GSH. These findings imply that andrographolide's antiatherogenic activity in rabbits was related not only to lipid profile modification, but also to anti-inflammatory and antioxidant actions.

2.7 Invivo antimalarial activity of *A. paniculata*

Widyawaruyanti *et al.*, 2014. Three phytopharmaceutical preparations of *A. paniculata* fractions containing diterpene lactones as an active ingredient were produced and tested for antimalarial activity against *Plasmodium berghei*. The oral treatment of the *A. paniculata* fractionation products twice a day for four consecutive days was used in an in vivo antimalarial experiment on *Plasmodium berghei* infected mice. The results showed that three phytopharmaceutical compounds of *A. paniculata* suppressed parasite development from 70.15 percent to 80.35 percent. There was no substantial difference in antimalarial activity between tablets II and III, although there was a significant difference between tablet I and tablets II and III. It was established that the antimalarial activity of *A. paniculata* active ingredient depends on the raw material shape.

2.8 Effect of *A. paniculata* on hyperglycemic mediated oxidative damage in renal tissues

Sivakumar *et al.*, 2015 evaluated the protective effect of *A. paniculata* ethanol extract on hyperglycemic mediated oxidative damage in the renal tissues of experimental diabetic rats and the potential bioactive components of *A. paniculata* ethanol extract were investigated using GC-MS techniques. The results showed that an ethanol extract of *A. paniculata* might protect experimental diabetic rats' renal tissues from hyperglycemic-mediated oxidative damage. According to India's ancient indigenous medical traditions, this plant possesses a variety of therapeutic properties without causing any serious side effects. This plant has the potential to be highly useful in the prevention of hyperglycemic problems and tissue damage caused by oxidation. These benefits are attributed primarily to its antioxidant characteristics, as evidenced by a strong quenching effect on the degree of lipid peroxidation, as well as an improved defense system.

2.9 Beneficial effects of an *A. paniculata* extract and andrographolide

Thakur *et al.*, 2016 examined the effects of a standardized *A. paniculata* extract and pure andrographolide on cognitive skills, oxidative stress, and cholinergic function in diabetic rats The findings in this communication clearly implied that *A. paniculata* and andrographolide might be a therapeutic alternative for the prevention or treatment of cognitive impairments that are common in diabetic patients. Methods for identifying the biological processes and mechanisms underlying their identified and diverse other effects could lead not only to novel pharmacological targets and strategies for the prevention and cure of diabetes-related comorbidities but also to a better understanding of the pharmacological principles underlying *A. paniculata's* widespread use in traditional medical systems.

2.10 Assessment of cytotoxicity of leaf extracts of *A. paniculata* and *Aspilia africana*

Ala *et al.*, 2018 investigated the cytotoxic effects of the two plants' aqueous, methanol, and chloroform extracts in a 1:1 mixture at varying concentrations on murine hepatocytes, thymocytes, and splenocytes in this work. Murine cells were planted in microtiter and tested with the mixed extracts at various doses, with cell viability measured using MTT assay. The extracts employed included a combination aqueous extract (CAE) of the plants and a combination chloroform extract (CCE). The percentage viability was minimal after 72 hr of incubation with a 500 g/ml extract concentration. Splenocytes, thymocytes, and hepatocytes treated with CAE showed viability values of 35%, 28%, and 64%, respectively, and with CCE, viability values of 26%, 26%, and 36%, respectively, comparison to the control group. According to the findings, the extracts were cytotoxic to murine cells at this concentration and incubation time. These low results imply that the extracts have cytotoxic interactions.

2.11 Quantitative HPLC analysis of andrographolide in *A. paniculata*

Meenu Sharma *et al.*, 2012 A simple, quick, selective, and quantitative HPLC technique for determining andrographolide in *A. paniculata* at two distinct phases of its life cycle was devised. Whole plant material was evaluated at two distinct phases, one after 110 days of planting, just before blooming (Sample A), and the other at crop maturity, bearing flowers and completely formed seed capsules (Sample B). The mobile phase was methanol: water (65:35), with a flow rate of 1.5 mL/min. Pure andrographolide was found to have a retention duration of 2.871 min. It was established HPLC technique may be used to determine the concentration of andrographolide in *A. paniculata* plant samples.

2.12 Isolation of PBMCs from whole blood with Ficoll Hypaque density centrifugation

Gill *et al.*, 2019 The researcher's study gibes an insight on peripheral blood mononuclear cells and the ficoll density gradient method. Peripheral blood mononuclear cells are frequently employed in research and therapeutic applications, and they are also a great tool for investigating numerous aspects of disease and biology in vitro. PBMCs include a variety of cells, including lymphocytes, monocytes, and macrophages, which are required by the immune system to fight diseases and pathogens.

2.13 Isolation of human PBMCs

Panda *et al.*, 2013 performed isolation of PBMCs using ficoll histopaque by density gradient centrifugation from whole blood. The protocol shows the step-by-step process to perform this procedure. In this procedure equal volume of PBS and whole blood is mixed. Whole blood: PBS: Ficoll is 1:1:0.7. The centrifugation has to be performed at 400 Xg when the temperature is fixed at 20℃. It also provides a list of materials and notes to follow for optimized isolation of monocytes from blood with the least amount of cell death.

2.14 Serum levels of IL-12 and the production of IFN-gamma, IL-2, and IL-4 by PBMC

Kovacs *et al.*, 2000 has studied the cytokines produced by PBMCs and the level of their production. They compared the effect of VA extract on blood levels and cytokine production in a group of cancer patients receiving treatment to healthy untreated controls. This study also focuses on IL-12 serum levels, as well as the production of IFN-gamma, IL-2, and IL-4 in PBMC of patients before therapy

CHAPTER 3

AIM AND OBJECTIVES

3.1 AIM

Our aim is to study the immunomodulatory effect of *A. paniculata* extract on human peripheral blood mononuclear cells.

3.2 OBJECTIVES

- Extraction and characterization of *A. paniculata* extract.
- In-vitro assessment of the cytotoxic effect of *A. paniculata* extract on Peripheral blood mononuclear cells (PBMCs).
- Evaluation of the possible immunomodulatory effect of *A. paniculata* extract on PBMC against Concanavalin A (Con-A) and Lipopolysaccharide (LPS).

CHAPTER 4

WORK PLAN

COMPLETED

Collection of plant sample (*A. paniculata*)

Submission of the plant for authentication

Solvent extraction

Characterization and optimization of sample

Collection of buffy coat

PBMC separation

Cytotoxicity assay

Lymphoproliferation assay

FUTURE WORKS

RNA extraction from PBMC to cDNA

Determination of cytokine level

CHAPTER 5

MATERIALS AND METHODS

5.1 SAMPLE PREPARATION

The plant was collected and prepared for undergoing extraction procedure.

5.1.1 Materials Required

1. Plant (*A. paniculata*)

2. Mortar and Pestle

5.1.2 Procedure

Plant *A. paniculata* (Acanthaceae), an herb collected from Madhavaram, Chennai, Tamil Nadu, India. The plant is air dried at room temperature for three days (Figure 5.1). The plants are then authenticated and taxonomically identified at the University of Madras, Guindy. The leaves of each plant are cleaned thoroughly to remove all debris, insects, and adhering sand particles. The samples are air-dried at room temperature until constant weights are obtained and then pulverized to powder using mortar and pestle. This powdered specimen is then stored at room temperature for further utilization (Augustine *et al*., 2014).

Figure 5.1: *A. paniculata*-Air dried at room temperature

A. paniculata dried for three days to decrease moisture content before being ground into powder for extraction.

5.2 SOLVENT EXTRACTION

This method is performed to extract the specific bioactive compound from the sample.

5.2.1 Materials Required

1. Powdered plant sample
2. Ultrasonicator
3. Distilled water

5.2.2 Procedure

The powdered plant sample is taken (20g). The extraction is carried out by the boiling method. Plant powder is dissolved in 200 ml (1:10) of distilled water. The sample was sonicated at different time intervals (2, 4, 6, 8, and 10 min) shown in Figure 5.2. The solution is then boiled at 65°C for 30 min. The

sample is cooled and centrifuged at 10,000 RPM for 15 min. The supernatant was taken. It was filtered using a syringe filter and stored for further analysis**.**

Figure 5.2: Solvent extracted by ultrasonication of sample

Ultrasonication of *A. paniculata* plant powder dissolved in distilled water (1:10 w/v).

5.3 OPTIMIZATION AND CONFORMATION OF SAMPLE

The optimization and confirmation of the sample is done by performing the following methods

5.3.1 High-performance liquid chromatography

HPLC instrument was used for chromatographic separation using the C-8 column. HPLC aims to confirm identify and quantify the compound andrographolide in the plant extract.

5.3.1.1 Materials required

1. HPLC methanol and water

2. Standard and sample solution

The stock solution of the Standard and sample were prepared to perform HPLC.

STOCK SOLUTION

1mg of andrographolide (standard compound) is dissolved in 50% ethanol Concentration-1mg/ml

STANDARD SOLUTION

Standard solutions (Figure 5.3) are prepared in five different concentrations from the stock solution.

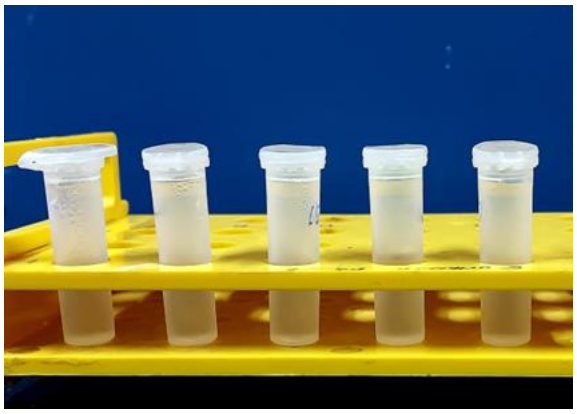


Figure 5.3: Andrographolide standard solution in different concentrations

Different concentration was prepared by dissolving pure market compound andrographolide in 50% ethanol (1:1 w/v) to determine the concentration sample in the extracted solvent.

Table 5.1: Standard preparation at the different concentrations for HPLC

STANDARD CONCENTRATION (µg/mL)	VOLUME OF STOCK (µl)	VOLUME OF ETHANOL (50%) (µl)
50	50	950
100	100	900
150	150	850
200	200	800
250	250	750

SAMPLE SOLUTION

The sonicated and filtered solvent prepared (Figure 5.4) using the above procedure was taken to perform HPLC.

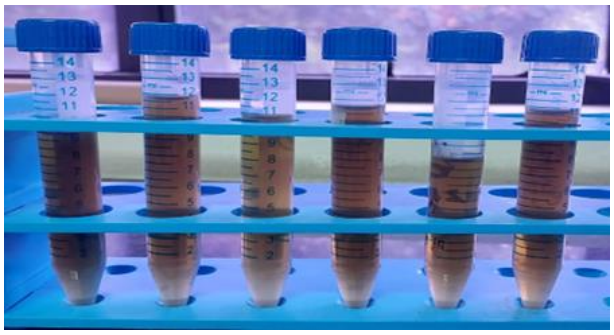


Figure 5.4: *A. paniculata* extract sonicated at different periods for HPLC

The sonicated extract of *A. paniculata* filtered using syringe filter to run on HPLC C-8 column.

5.3.1.2 Procedure

- The standard samples and solvent samples were injected into HPLC.
- HPLC analysis for each sample and solvent was continued for 7 min.
- The retention time of andrographolide was 4-4.5 min
- HPLC analysis for the standard solution of specific concentration was carried and solvent sonicated for different periods were carried out 3 times (Sajeeb *et al*., 2015).

5.3.2 Fourier transform infrared spectroscopy

To confirm the presence of the compound andrographolide in the *A. paniculata* extract

5.3.2.1 Principle

The instruments create an IR irradiation beam generated by a burning black-body source. Following that, the beam goes via an interferometer, where spectral encoding occurs. The beam then enters the sample compartment, where the sample absorbs certain frequencies of energy that are unique to the sample as determined by the interferogram.

The detector then detects the unique interferogram signal in energy against time for all frequencies at the same time. The appropriate spectrum was achieved after the interferogram used fourier transformation computer software to automatically remove the background spectrum from the sample spectrum (Figure 5.5).

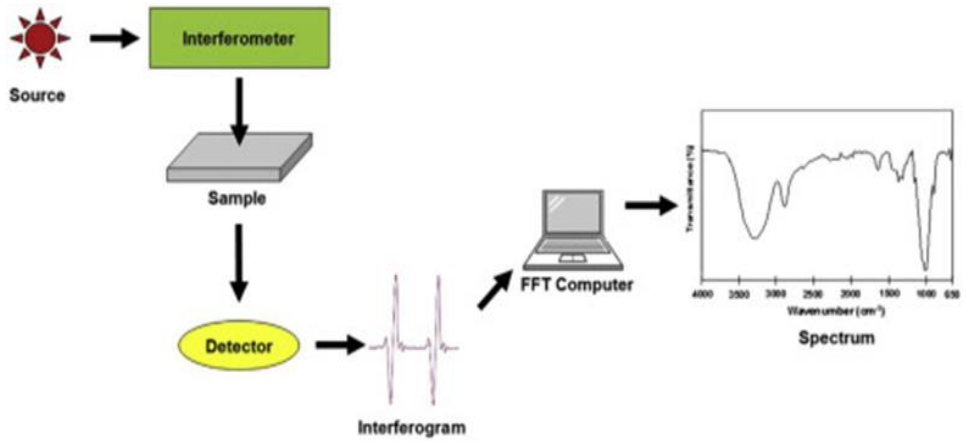


Figure 5.5: Fourier transform infrared spectrophotometer

Block diagram of the working process of FTIR with its components. (Undavalli *et al*., 2021)

5.3.2.2 Procedure

- Both the standard (andrographolide) and the *A. paniculata* extract are in liquid form.
- The liquid sample is placed in between pairs of polished sodium chloride or potassium bromide plates (salt plates).
- Only a drop of liquid is placed in the plate to form a thin layer for infrared light to pass through
- The spectrometer focuses IR beams on the sample and detects the range of the beam, and the frequencies, the sample absorbs.
- The sample must be thin enough for infrared light to pass through.
- Then the pair of plates is inserted into the spectrophotometer.

5.3.3 Nuclear magnetic resonance spectroscopy

To determine the purity and structural confirmation of the sample andrographolide.

5.3.3.1 Principle

Nuclear magnetic resonance spectroscopy (NMR spectroscopy) is a technique that uses the magnetic properties of specific atomic nuclei to determine the physical and chemical properties of atoms or molecules. The method can provide detailed information about a molecule's structure, dynamics, reaction state, and chemical environment. The intramolecular magnetic field surrounding an atom in a molecule changes the resonance frequency, allowing access to details of a molecule's electronic structure and individual functional groups.

5.3.3.2 Procedure

- Place the sample in a magnetic field and excite the nuclei sample into nuclear magnetic resonance with the help of radio waves to produce NMR signals.
- These NMR signals are detected with sensitive radio receivers.
- This gives details of a molecule’s individual functional groups and its electronic structure.
- Nuclear magnetic resonance spectroscopy is a conclusive method of identifying mono molecular organic compounds.
- This method provides details of the reaction state, structure, chemical environment, and dynamics of a molecule.

5.4 PBMC ISOLATION

To perform isolation of PBMC from whole blood using density gradient centrifugation.

5.4.1 Principle

A ficoll-hypaque solution, when prepared, has a specific gravity at room temperature that is denser than lymphocytes, monocytes, and platelets but less dense than granulocytes and red blood cells, allowing for successful separation of these cell populations. Because they have a lower density, mononuclear cells and platelets collect on top of the ficoll-hypaque layer; in contrast, red blood cells and granulocytes have a higher density than ficoll-hypaque and collect at the bottom of the ficoll-hypaque layer.

5.4.2 Materials required

1. Whole blood
2. 1X PBS
3. Ficoll
4. Centrifuge
5. Falcon tube
6. Pipette
7. RPMI media

5.4.3 Procedure

- 10ml of whole blood was drawn and an equal volume of 1X PBS was added (1:1)
- 3.5ml of ficoll was taken in a separate falcon tube.
- Diluted blood was gently layered over ficoll in two separate falcon tubes.

- It is Centrifuged at 400 Xg for 30min at 20℃.
- PBMC (Figure 5.6) was carefully harvested by directly inserting the pipette to mononuclear cell layer.

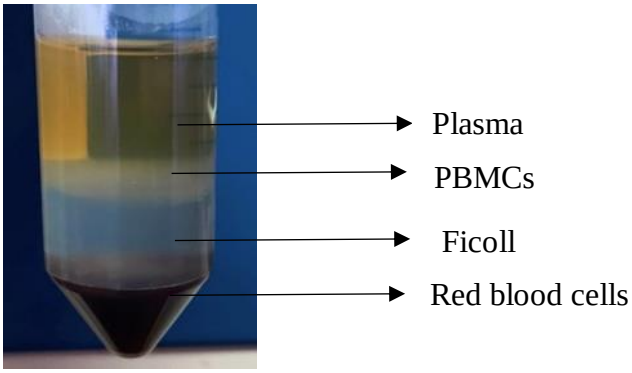


Figure 5.6: PBMC separation using ficoll gradient centrifugation

The whole blood and PBS after ficoll gradient centrifugation get separated into 4 layers visible in falcon tube.

- The harvested cell is washed for four times at 200Xg for 10 min at 20℃ with 3 volumes of 1X PBS.
- The pellet obtained after wash is suspended in RPMI media. (Panda *et al*., 2013).

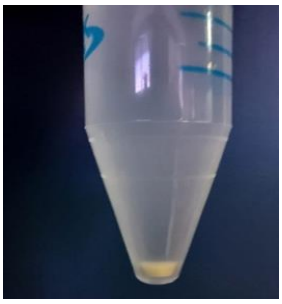


Figure 5.7: PBMC pellet obtained after PBS wash

After 3 PBS washes, the supernatant was discarded leaving a whitish colored PBMC pellet which is then suspended in RPMI media.

5.5 CELL VIABILITY

The cell viability was checked to determine the number of cells to be plated in each well of 96-well plates.

5.5.1 Trypan blue exclusion assay

The test is used to determine the number of viable cells present in a cell suspension to perform cytotoxicity assay.

5.5.1.1. Principle

Dead cells lack complete cell walls that prevent some dyes from entering, such as trypan blue, eosin, or propidium. In this test, a cell suspension is simply combined with dye and visually inspected to see if the cells absorb or reject the dye. A live cell will have a clear cytoplasm in this technique, while a nonviable cell will have a blue cytoplasm**.**

5.5.1.2 Materials required

1.Trypan blue

2. Hemocytometer

3. Microscope

4. Glass slide

5. PBMC pellet obtained after wash

5.5.1.3 Procedure

- 1 part of 0.4% trypan blue and 1 part cell suspension (dilution of cells) is mixed
- The mixture is allowed to incubate for 3 min at room temperature.
- Mixing is performed in a well of a microtiter plate or a small plastic tube using 10 to 20 µl each of cell suspension and trypan blue.
- A drop of the mixture of trypan blue and cell mixture is added to a hemocytometer.
- The hemocytometer is placed and viewed under a microscope (Figure 5.8).
- The unstained cells (viable cells) are counted.
- Stained (nonviable) cells are counted separately in the hemocytometer.
- The percentage of viability of cells is calculated as follows (Strober *et al*., 2015)

5.5.1.4 Calculation of cell viability

$$\text{Cell viability (\%)} = \frac{total\ number\ of\ viable\ cells}{Total\ number\ of\ cells} \text{x}100$$

The above formula is used to calculate the cell viability percentage. The total number of viable cells is counted using a hemocytometer (Figure 5.8). Further experiments are carried out only if the cell viability percentage is greater than 95%.

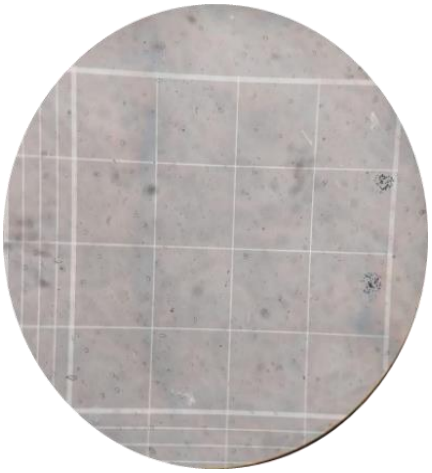


Figure 5.8: Cells visualized on the hemocytometer under the microscope at 40X magnification

Cell count performed by suspending cells in equal volume of trypan blue visualized under hemocytometer to calculate cell viability percentage. The number of cells per ml calculated to determine the number of cells be plated in each well

5.6 CYTOTOXICITY ASSAY

To measure the ability of cytotoxic compounds to cause cell death or damage

5.6.1 Principle

Trypan blue is a stain that only labels dead cells, allowing for the quantification of live cells. Trypan blue cannot cross the cell membrane of living cells and enter the cytoplasm because they have an unbroken cell membrane. Trypan blue enters the cytoplasm of a dead cell through the permeable cell membrane. Only dead cells have a blue colour under light microscope investigation.

5.6.2 Materials required

1. Trypan blue
2. Hemocytometer
3. Microscope
4. Glass slide
5. PBMCs
6. Control-Concanavalin-A(Con-A) and LPS
7. Plant extract in different concentrations
8. 96-well plate

5.6.3 Procedure

- PBMCs were isolated from whole blood by density gradient centrifugation.
- The viability of cells and number of cells per ml was calculated using trypan blue exclusion assay.
- Approximately 12,000 cells were seeded into each well of the 96 well plate
- The cells were seeded into a 96-well plate to check the effect of PBMCs incubated with the plant extract of *A. paniculata* for 24 and 48 hr. in the presence of Con-A (1 µg/mL) or LPS (1 µg/mL) as control.
- The cell viability was checked by performing with trypan blue exclusion assay by following the procedure as mentioned in 5.5.1.

5.7 LYMPHOPROLIFERATION ASSAY

To study the lymphocyte proliferation when exposed to different concentrations of *A. paniculata* extract by performing MTT (3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide)assay.

5.7.1 Principle

The MTT assay is a colorimetric assay used to measure the metabolic activity of cells. The ability of nicotinamide adenine dinucleotide phosphate (NADP)-dependent cellular oxidoreductase enzymes to reduce the tetrazolium dye MTT to its insoluble formazan, which has a purple color.

5.7.2 Materials required

1. MTT reagent
2. PBMCs
3. Plant extract in different concentrations
4. 96-well plates
5. Control-Concanavalin-A(Con-A) and LPS

5.7.3 Procedure

- PBLs are isolated by gradient centrifugation with Ficoll– Hypaque solution.
- The viability of cells was more than 95% determined by the trypan blue dye exclusion test.

- The effect of the extracts on the PBLs () in the presence or absence of T lymphocyte mitogens (1 µg/mL of Con A) or B lymphocyte mitogen (1 µg/mL of LPS) with *A. paniculata* in a total volume of 200 µl/well at the same conditions for 24 and 48 hr.
- They were further incubated for 4 h (Figure 5.9) with 10 µL of 5 mg/mL MTT.
- The isolated PBLs and the compound is added in 96-well plates containing a final volume of 200 µl/well.

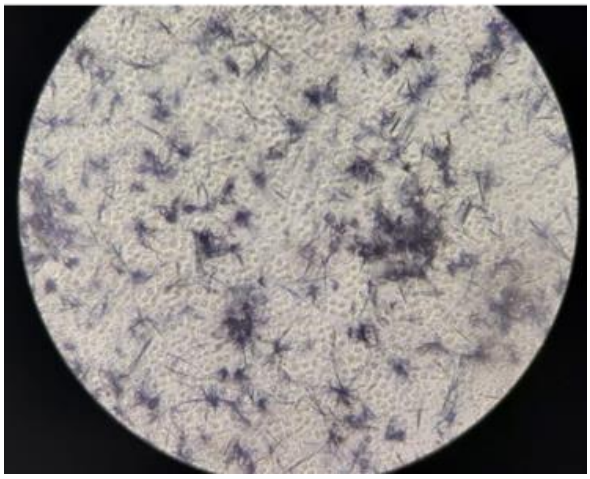


Figure 5.9: Formazan crystal formation after 4hr of incubation with MTT solution viewed under a microscope at 40 x magnification

Microscopic view of MTT formazan crystals formed when PBMCs were treated for 24 and 48 hr with various concentrations of *A. paniculata* extracts. The photo was shot at a magnification of 40x.

- 10 µl MTT Solution is added and it is incubated for 4 hr at 37°C.
- After incubation the 96 well plate centrifuged at 4500 RPM for 10 min at 20°C and the supernatant is discarded.

- To the pellet 100 µl Solubilization solution (DMSO) is added to each well to dissolve formazan crystals. It is mixed to ensure complete solubilization.
- Absorbance was taken on an ELISA reader at 450-630 nm (Glicksman et al., 2004).

CHAPTER 6

RESULTS AND DISCUSSION

6.1 CONFIRMATION OF THE PLANT

The herbarium specimen *A. paniculata* submitted to the Centre for Advanced Studies in Botany herbarium, University of Madras was identified as *A. paniculata* Nees.

6.2 SOLVENT EXTRACTION

The leaves of the plant *A. paniculata* were air-dried at room temperature for 3 days. The leaves of the air-dried plant are powdered using mortar and pestle and extracted using distilled water (1:10). The mixture is ultrasonicated at different time periods (2, 4, 6, 8, and 10 min) and boiled at 65℃ for 30 min for extraction of bioactive compound andrographolide. The supernatant was collected after centrifuging at 10000 RPM for 15 min. The sample is stored at -20℃ for further analysis.

6.3 OPTIMIZATION AND CONFIRMATION OF SAMPLE

The optimization and confirmation of the sample is done by performing HPLC, FTIR spectroscopy, and NMR spectroscopy.

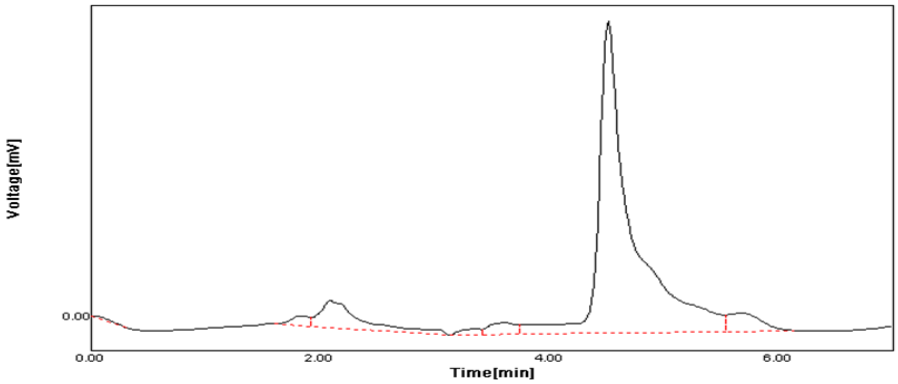
6.3.1 Optimization and confirmation of sample and standard using UV Spectroscopy.

The spectrum reading for the standard andrographolide (1mg of andrographolide in 1ml 50% ethanol) was scanned using UV Visible Spectrophotometer in the range of 200 nm to 500 nm using 50% ethanol as the blank solution. Similarly, the spectrum reading for the above-mentioned A. paniculata sonicated and unsonicated samples were read at 200 nm to 500 nm using water as the blank solution. However, the readings obtained were not accurate. Hence HPLC was performed for the confirmation of the presence of andrographolide by comparing with standard (Meenu *et al*., 2012).

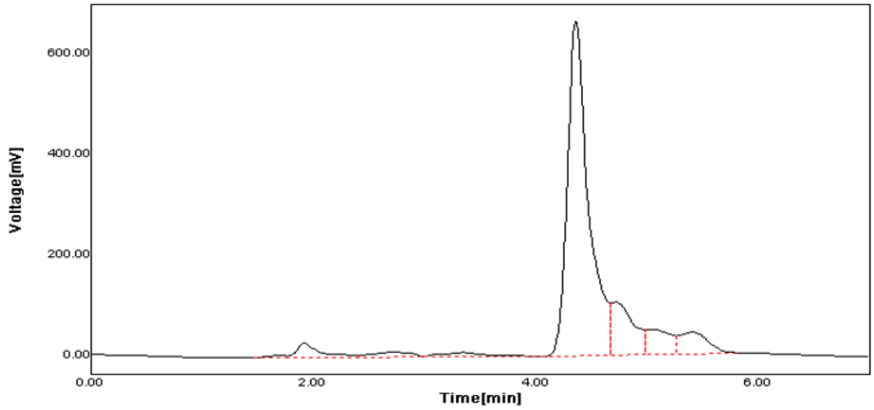
6.3.2 Optimization and confirmation of sample and standard using HPLC

6.3.2.1 HPLC Standard reading

The standard (andrographolide) solution was prepared in different concentrations. The experiment was performed in triplicates in the HPLC-C8 column and the chromatogram was obtained (Figure 6.1).


Voltage[mV]
0.00
0.00
2.00
4.00
6.00
Time[min]

A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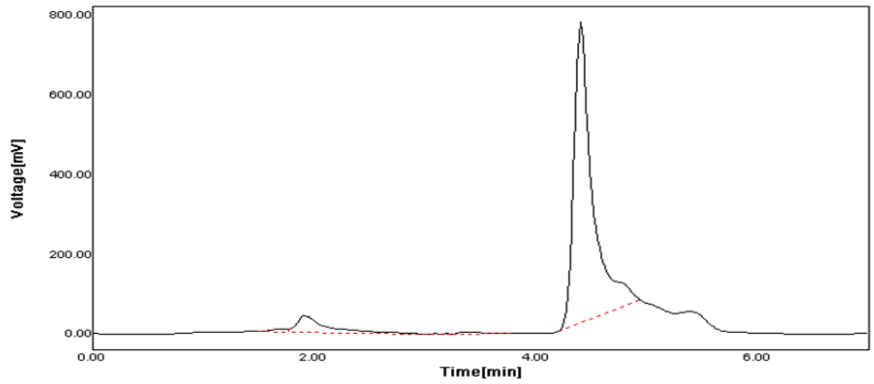
200.00
0.00
Voltage[mV]
0.00
2.00
4.00
6.00
Time[min]

B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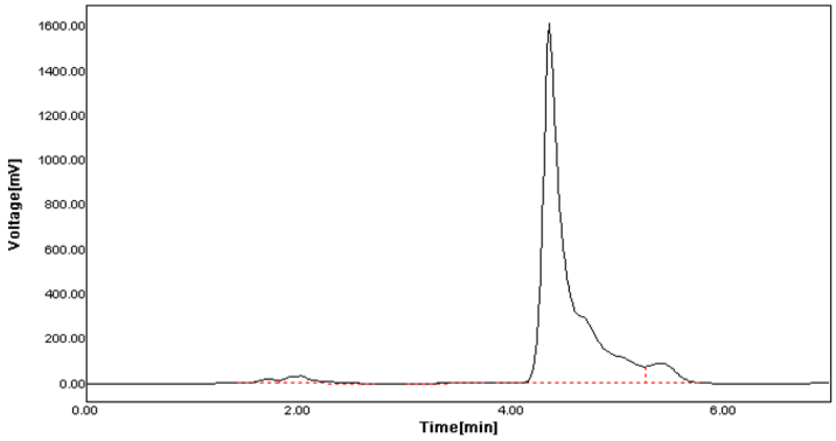
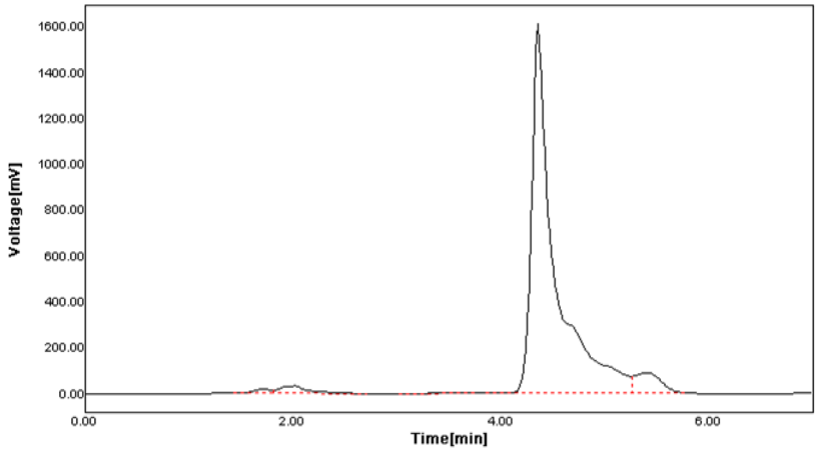
600.00
400.00
200.00
0.00
Voltage[mV]
0.00
2.00
4.00
6.00
Time[min]

C



D



E

Figure 6.1: HPLC Chromatograms of different concentration of standard (A) 50 µg/mL of standard andrographolide, (B) 100 µg/mL of standard andrographolide, (C) 150 µg/mL of standard andrographolide, (D) 200 µg/mL of standard andrographolide, and (E) 250 µg/mL of standard andrographolide

The retention time of andrographolide was 4-4.5 min. The chromatogram of different concentrations of the sample depicts the elution time and amount of compound extracted Eluent used is Methanol: Water (65:35, v/v); Detection at 225 nm.

The area under the curve for the standard andrographolide is represented in the Table 6.1.

Table 6.1: Concentration of standard andrographolide vs area under the curve

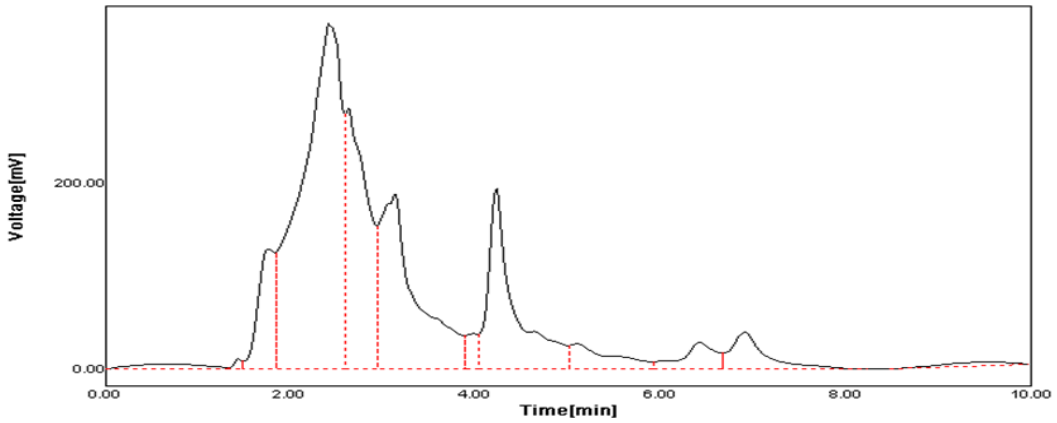
Concentration (µg/mL)	Area under the curve (mVs)
50	2965
100	9377
150	10184.667
200	11463
250	24085.33

The experiment was carried out in replicates. The values in the above table represents the average of the values

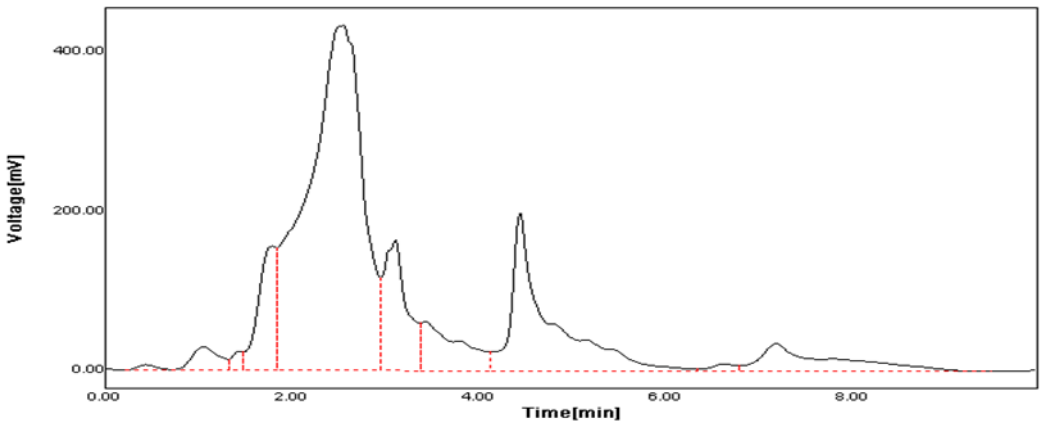
6.3.2.2 HPLC sample reading

The sample (plant extract of *A. paniculata*) prepared was run on the HPLC-C8 column using methanol (65 volumes) and water (35 volumes) as the mobile phase (Meenu et al., 2012). The chromatogram of the unsonicated and sonicated sample were obtained (Figure 6.2). Chromatogram results reveal the extraction of bioactive compound andrographolide from a plant extract of *A. paniculata* ultrasonicated for specific time. HPLC was performed in the C8 column where elution is carried in Isocratic mobile phase Methanol: Water

(65:35, v/v); Detection: 225 nm. The chromatogram depicts time of elution and the amount of andrographolide extracted for a specific sonicated time. The area under the curve for the sonicated sample is represented in the Table 6.2.



A



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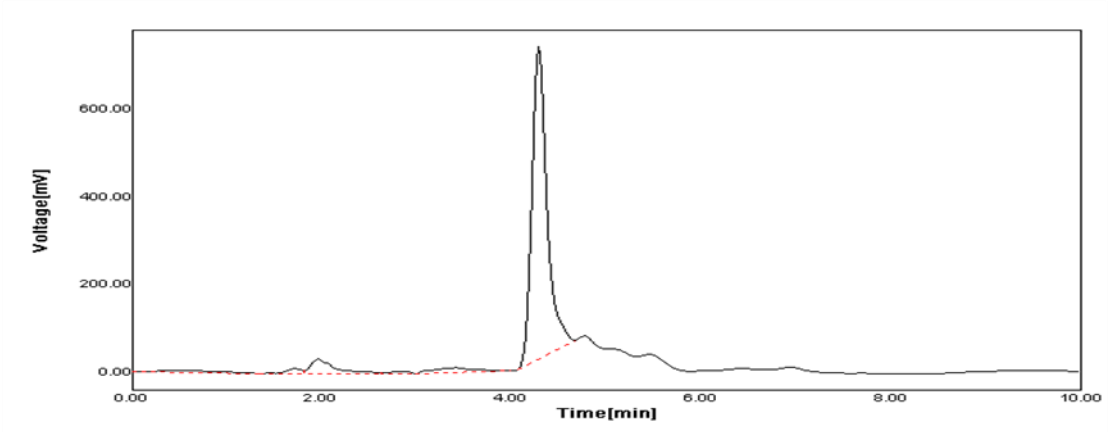
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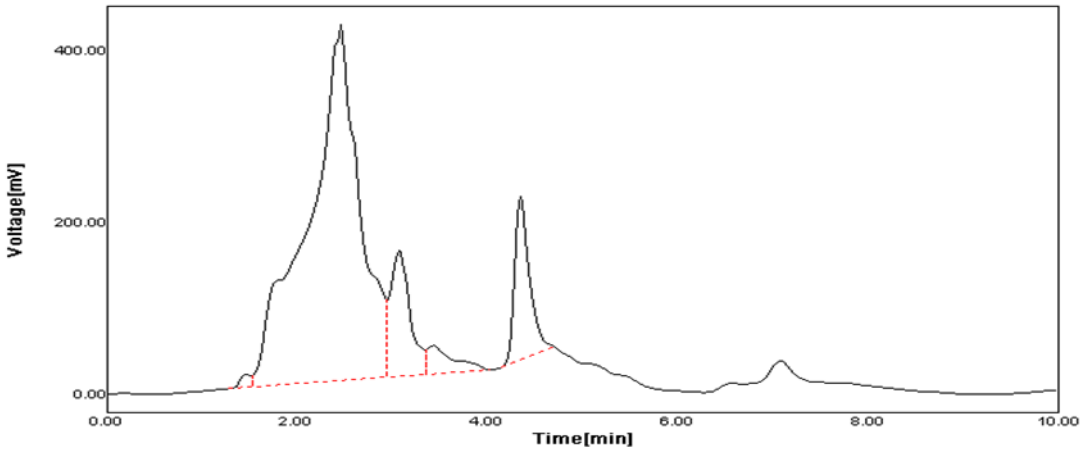
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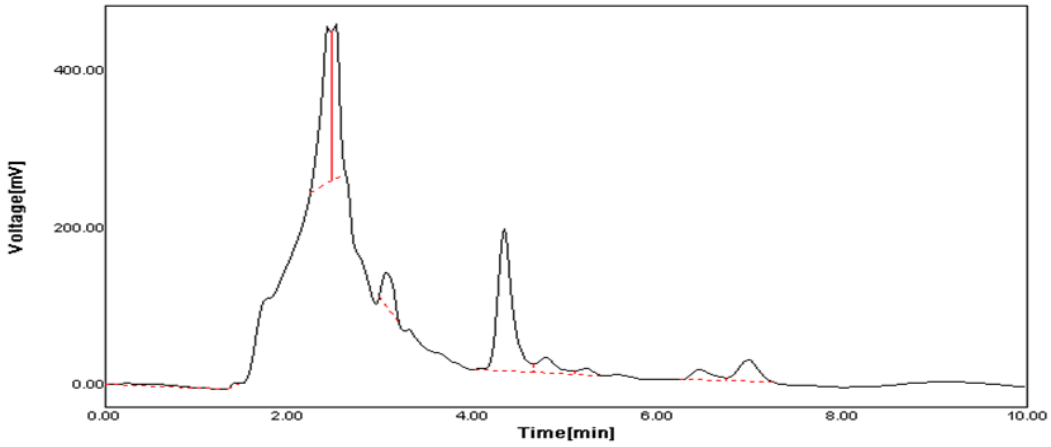
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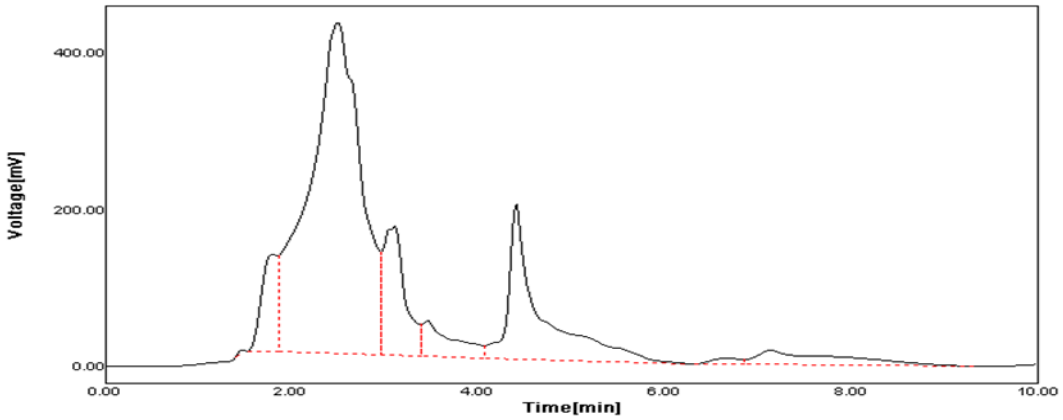

F

Figure 6.2: HPLC Chromatograms of the sonicated sample for (A) 0 min, (B) 2 min, (C) 4 min, (D) 6 min, (E) 8 min, and (F) 10 min. having retention time at 4-4.5 min

Table 6.2: Tabulation of the sample (extract) reading in HPLC at absorbance 225 nm

Sonication time (min)	Time of elution (min)	Area under the curve (mVs)
0	4.43	3793.70
2	4.28	5389.80
4	4.35	7900.07
6	4.33	2049.81
8	4.41	1994.02
10	4.23	4601.13

Experiment was carried out in replicates. The values in the above table represents the average of the values

The identification, measurement, and comparative analysis of standard andrographolide and sample were used to standardize *A. paniculata* preparations. The findings revealed that a high amount of andrographolide is present in 4 min sonicated sample as compared to the standard andrographolide. The values obtained in Table 6.2 were then plotted into a standard graph to find the concentration of andrographolide present in the 4 min sonicated sample. Figure 6.3 shows the concentration vs area under the curve that was plotted for obtaining the concentration of andrographolide in the plant extract. The value obtained from the equation y = mx + c determines the concentration of andrographolide in the sample.

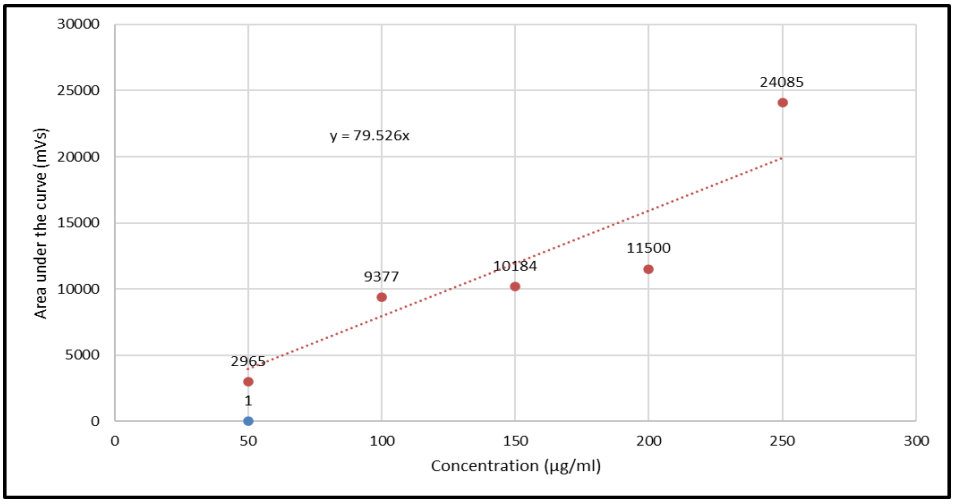

Figure 6.3: HPLC Standard graph

(Concentration vs area under the curve)

Standard graph plotted to determine the concentration of the sample using the y=mx+c equation.

The standard curve (Figure 6.3) was generated by plotting the concentration of andrographolide vs area under the curve obtained by performing HPLC of the ultrasonicated samples (plant extract of *A. paniculata*)

From the Standard graph (Figure 6.3),

$$y = mx+c$$

$$y= 79.526x$$

Area under the curve (4-min sample) [y] = 7900.0684

$$7900.0684 = 79.526 \times x$$

$$x = 99.34\ \mu g/mL$$

Concentration of sample(extract)=99.34 µg/mL

Thus, from the above equation, the concentration of the 4 min ultrasonicated sample is found to be 99.34 µg/mL.

6.3.3 Fourier transform infrared spectroscopy

FTIR was performed to confirm the molecular composition and structure of the standard and extract. *A. paniculata* extract and andrographolide contain amino acids, amides, amines, carboxylic acid, carbonyl compounds, and organic hydrocarbons functional groups (Doss *et al*., 2019).

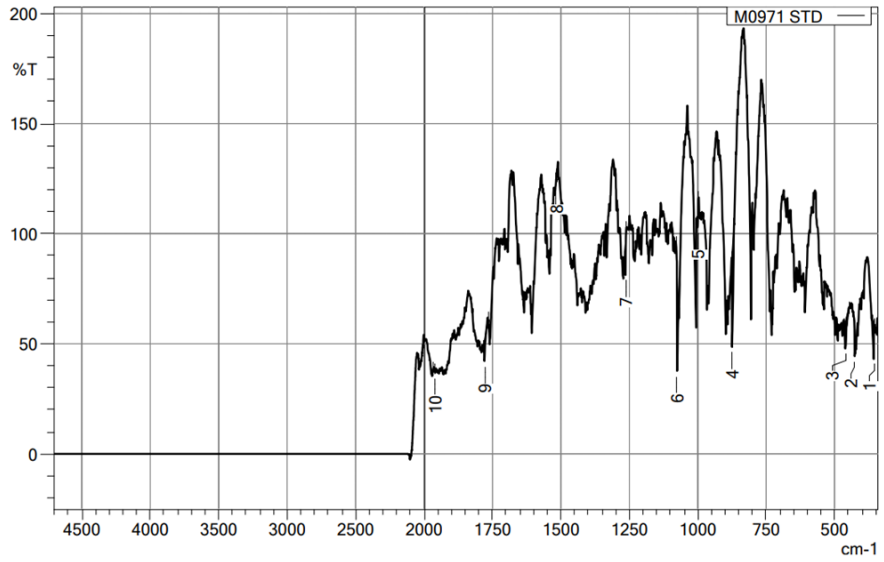

Figure 6.4: IR spectra of standard andrographolide

FTIR spectra of andrographolide dissolved in 50% ethanol (1:1 w/v) indicating the presence of functional groups and phytochemical constituents.

The IR spectra of standard andrographolide (Figure 6.4) show absorption bands rising from the vibrations of C-O-C (1075.54 cm^{-1}) and C-OH (1264.08 cm^{-1}) indicating the presence of the ether and carboxy group respectively. The absorption bands between 1700 cm^{-1} and 1500 cm^{-1} have been described as various strong carbonyl i.e., C=O amide peaks due to residual organic groups. anhydride shows an intense characteristic absorption band between 1780 cm^{-1} and 1740 cm^{-1}. To be more specific, 1778.99 cm^{-1} absorbance indicates the presence of anhydride The absorption band 1959.68 cm^{-1} identifies the presence of strong stretching allene (C=C=C). The intensity of the C–C absorbance group decreases with increasing aging time, while the intensity of the broad absorbance band in the C-O stretching 1200-1800 cm^{-1} region increases respectively with aging time.

By comparison with the functional groups present in the andrographolide, the market product which we used in the experiment is confirmed to be andrographolide ($C_{20}H_{30}O_5$).

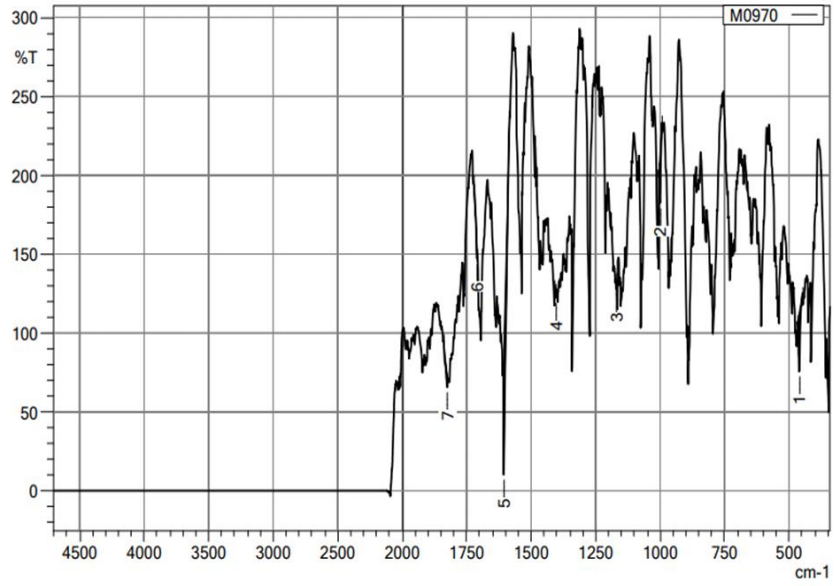

Figure 6.5: IR spectra of sample (Extract of *A. paniculata*)

FTIR spectra of sample containing natural compound andrographolide indicating the presence of similar functional groups and phytochemical constituents.

The FTIR spectra of the sample (plant extract of *A. paniculata*) in Figure 6.5 shows the absorption bands rising from the vibrations of C-O stretching aliphatic ether (1169.81 cm^{-1}) and C=C aromatic alkene (1606.17 cm^{-1}). The absorption band 1959.68 cm^{-1} (C=C) identifies the presence of strong stretching α, and β-unsaturated ketone. The absorbance band 1710.43 cm^{-1} indicates the presence of stretching carboxylic acid dimer. The intensity of the C=O absorbance group decreases with increasing aging time, while the intensity of

the broad absorbance band in the C-C stretching 1100 – 1800 cm^{-1} region increases respectively with aging time.

The peak and shoulders exhibited in Figure 6.4 and Figure 6.5 are the results of functional group absorption in the tested samples. The FTIR spectra from graphs indicated identical peaks, which may be interpreted as a similar profile in chemical components. Andrographolide contains anhydride groups that can be seen in both samples. Other chemical constituents of the compound like lactone(C=C) are visible in both FTIR spectra Figure 6.5. From this, it is observed that the sample contains the bioactive compound andrographolide through comparative analysis.

6.3.4 Nuclear magnetic resonance spectroscopy

NMR spectroscopy was performed to identify the structural information of the compound andrographolide.

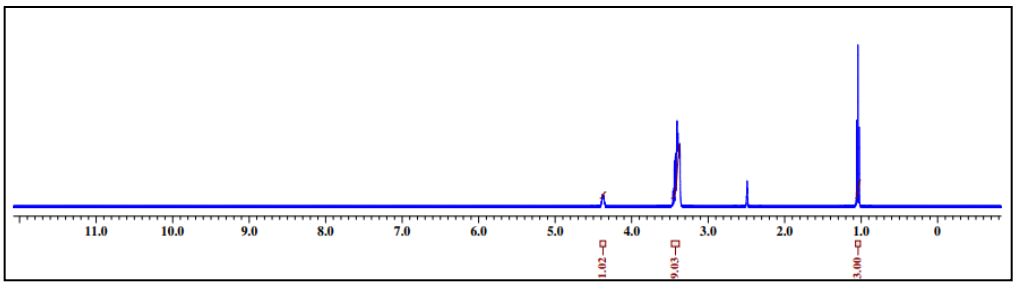

Figure 6.6: NMR spectra of the standard-Andrographolide

NMR spectra indicating the presence of phenolic group in compound andrographolide

Figure 6.6 shows the NMR spectroscopy results taken for the standard andrographolide. The reducing peak intensities at 4.3 to 4.4 ppm correspond to the presence of the phenolic group. The increasing peak intensities at 1.0 to 1.1 indicate the presence of an acetylenic group. The NMR signals of ether were observed at 3.3 to 3.5 ppm.

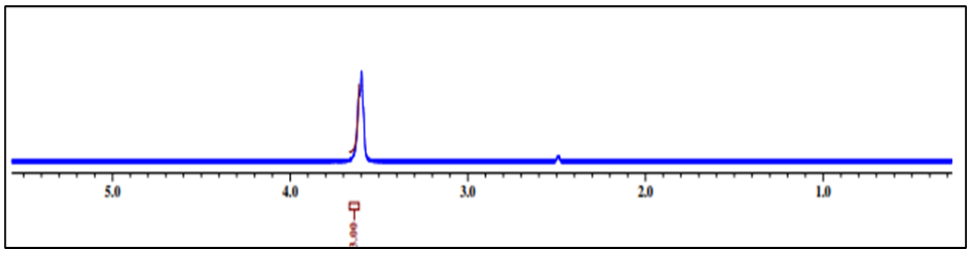

Figure 6.7: NMR spectra of solvent-*A. paniculata* extract

NMR spectra confirming the presence of phenolic group in *A. paniculata* extract

Figure 6.7 depicts the graphical representation of the NMR spectroscopy results obtained for the sample. The peak observed be 4.3 and 4.5 ppm indicate the presence of the phenolic group. A similar rise in peak can be observed in the standard in Figure 6.6. This indicates the presence of the same group in both the standard and sample.

6.4 ISOLATION OF PBMC (PERIPHERAL BLOOD MONONUCLEAR CELLS).

PBMCs were isolated from a human blood sample using ficoll density gradient centrifugation.

Table 6.3: Tabulation of different trials of Isolation of PBMC

S.NO	EXPERIMENT	OBSERVATION	RESULT
1.	1. 10ml whole blood + 10 ml 1x PBS + 10 ml ficoll (1:1:1) 2. Centrifugation at 1600 RPM for 40 min at 4℃	No phase layer separation was observed	No buffy coat obtained
2.	1. 10ml whole blood + 10 ml 1x PBS + 7 ml ficoll (1:1:1.15) 2. Centrifugation at 400 Xg for 30 min at 20℃	Phase layer separation was observed but not well defined	Buffy coat was not obtained.
3.	1. 10ml whole blood + 10 ml 1x PBS + 7 ml ficoll (1:1:0.7) 2. Centrifugation at 400 Xg for 30 min at 20℃	Well-defined phase layer separation was observed	Buffy coat was obtained.

Initially, isolation was performed by taking equal ratios of blood, 1X PBS, and ficoll (1:1:1), and the observation was recorded (Table 6.6). Different experimental trials were carried out under varying conditions to produce well-defined phase layers and obtain the buffy coat.

Thus, from table 6.3, it can be inferred that the layer separation was well-defined and the buffy coat was obtained with the ratio of blood, 1X PBS, and ficoll at 1:1:0.7. This was then washed three times with 1X PBS to get the pellet. The pellet was resuspended in supplemented RPMI-1640 media and incubated at 5% CO_2 and 37℃. The PBMC was viewed under inverted microscope (Figure 6.8).

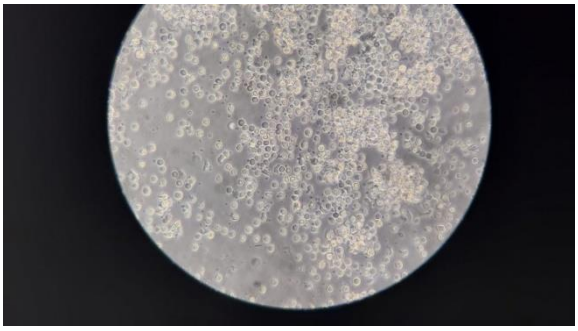

Figure 6.8: Microscopic view of PBMCs. (40X magnification).

Representative bright field microscopy images of PBMC on modified surfaces.

6.5 CALCULATION OF CELL VIABILITY

10 μl of 1:1 ratio of cell suspension and trypan blue was loaded on the hemocytometer and the total cell count was obtained.

$$\text{Cell viability (\%)} = \frac{\text{Total number of viable cells}}{\text{Total number of cells}} \text{x}100$$

Total number of viable cells =316+280+323+298=1217

Total number of dead cells =8

Total number of cells = 316+280+323+298+8=1225

By applying in the formula

$$\text{Cell viability (\%)} = \frac{1217}{1225} \text{x}100$$

$$\text{Cell viability (\%)} = 99.34\%$$

As cell viability was greater than 95% further experiments were performed.

6.6 CYTOTOXICITY ASSAY

In the present study we evaluated the cytotoxicity of *A. paniculata* extract induced *in vitro* in PBMCs. PBMCs ($12x10^4$ cells) are diluted in complete RPMI media in each well of 96 well plates and are incubated with extract for 24 and 48 hr. The sample was added in various concentration (1-10, 15, 20, 25, 30, 35, 40, 45, 50 µg/mL). The proliferation was determined by performing a trypan blue assay.

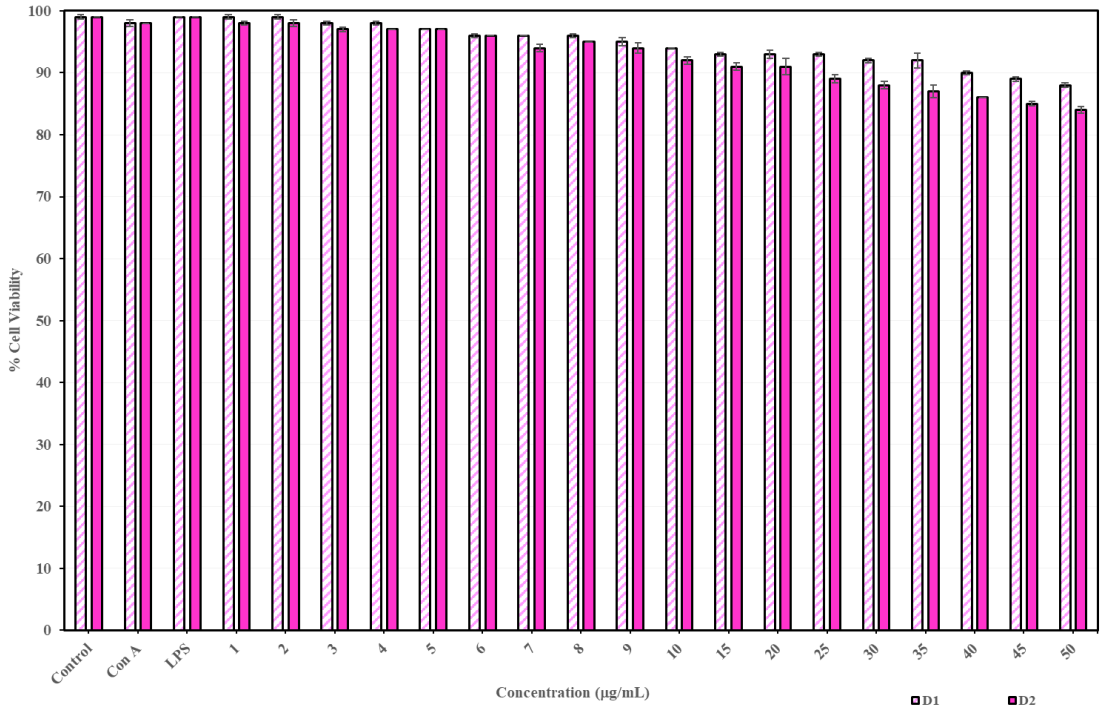

Figure 6.9: Evaluation of cytotoxicity using trypan blue exclusion assay

Cell viability evaluated in the trypan blue assay for PBMC incubated with different concentration of extract incubated at 37°C for 24 and 48 hr. Cells diluted in complete RPMI (RPMI-1640 medium supplemented with 10% FBS and 1% penicillin). Complete RPMI was used as the blank and Con A or LPS (1 µg/mL) was added to the RPMI in wells corresponding to control (Con A or LPS + RPMI). Each point represents the mean ± SE for the three experiments. The result in the figure depicts the cell viability cytotoxicity of *A. paniculata* extract.

Figure 6.9 shows relative death of cells with an increase in the concentration of extract. Even at the highest concentration (50 µg/mL) 88% of cells are viable in 24 hr incubation and 84% of cells are viable in 48 hr incubation. Hence, we can confirm the absence of cytotoxicity in the *A. paniculata* extract.

6.7 LYMPHOPROLIFERATION ASSAY

The results for the lymphoproliferative activity on PBMCs when treated with extract was identified by performing MTT assay. By performing this assay, the effect of *A. paniculata* on human peripheral mononuclear cells (PBMCs) was evaluated.

The results have been depicted in the graph (Figure 6.10). There was not time dependant inhibition of cell viability. No difference in cell viability was observed for cells treated with control (Con-A or LPS) and cells treated with the extract. Even at high concentrations, the cell viability was greater than 80%.

Thus, our results (Figure 6.10) depict that the compound andrographolide present in the plant extract interferes with lymphocyte proliferation and thus has immunomodulatory activity.

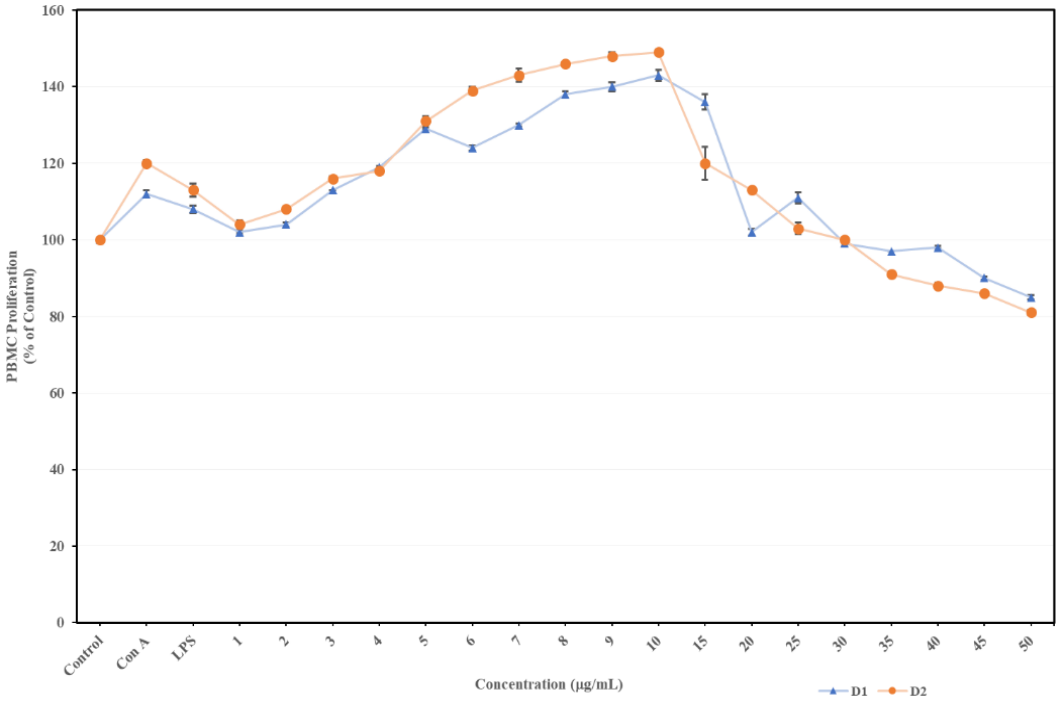

Figure 6.10: Cells incubated with different concentrations of *A. paniculata* extract quantified by MTT assay

MTT OD values for PBMCs ($12x10^4$ cells) incubated with different concentrations of extract ranging from 1 µg/mL to 50 µg/mL cells/ml. Cells diluted in complete RPMI (RPMI-1640 medium supplemented with 10% FBS and 1% penicillin) used to dilute the cells. Complete RPMI was used as the blank and an equal amount of Con A or LPS (1 µg/mL) was added to the RPMI in wells corresponding to control (Con A or LPS + RPMI). The graph is representation of three independent experimental data Values are Mean ± SE for a set of three values with their standard errors

CHAPTER 7

CONCLUSION

In this study, a simple and effective method was used for the extraction bioactive compound andrographolide from *A. paniculata*. Performing ultrasonication to the boiled *A. paniculata* extract exhibited an extraction of ≈100 μg/ ml of bioactive molecule-andrographolide. Furthermore, the extracted andrographolide was found to be less cytotoxic. The andrographolide extract also stimulated the lymphocytes proliferation against Con A or LPS. Thus, the andrographolide extracted from *A. paniculata* has immunomodulatory effect and can be used as herbal medicine which protects humans from pathogens by increasing immunity.

CHAPTER 8

FUTURE WORKS

The effect of the plant extract on PBMC has induced the lymphocyte proliferation can further be confirmed by quantifying IL-2 and IFN-γ production. For determining the cytokines such as IL2 and IFN-γ cytokine profiling is to be performed. PBMCs incubate with different concentration of extract incubated for 24 and 48 hr is to be centrifuged to obtain the pellet which is then used to isolate mRNA. Trizol extraction method is to be used for isolating mRNA. The obtained mRNA will then be converted to cDNA which is then subjected to RT PCR with specific primers. The obtained products are then visualized under UV Spectrophotometer using gel electrophoresis.

Molecular docking technique has to be performed to find the binding affinity of compound andrographolide. Chemical constituents and structure of the compound are to be taken from known literature sources. Molecular docking experiments are to be performed using the PyRx 0.8 software which will remove the water molecule as well as the ligands. This software will interact with andrographolide and determine the immunomodulatory effect of the compound.

CHAPTER 9

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